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T-bet Regulates the Terminal Maturation and Homeostasis of NK and Vα14*i* NKT Cells

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Summary

Natural killer (NK) and CD1d-restricted Vα14*i* natural killer T (NKT) cells play a critical early role in host defense. Here we show that mice with a targeted deletion of T-bet, a T-box transcription factor required for Th1 cell differentiation, have a profound, stem cell-intrinsic defect in their ability to generate mature NK and Vα14*i* NKT cells. Both cell types fail to complete normal terminal maturation and are present in decreased numbers in peripheral lymphoid organs of T-bet$^{-/-}$ mice. T-bet expression is regulated during NK cell differentiation by NK-activating receptors and cytokines known to control NK development and effector function. Our results identify T-bet as a key factor in the terminal maturation and peripheral homeostasis of NK and Vα14*i* NKT cells.

Introduction

The innate immune system plays a critical early role in host defense in response to viruses, bacteria, and tumor cells. Two of its cell lineages, NK and NKT cells, do not require prior sensitization for effector function and are vital both for initially combating infection and for subsequently activating the adaptive immune system by physical interaction with dendritic cells and by the secretion of immunoregulatory cytokines.

Although some progress has been made in identifying and understanding the in vivo stages of NK cell lineage commitment and maturation, there is currently little known about the precise molecular events that occur. NK cells develop from a common T/NK precursor bone marrow (BM) stem cell. The earliest known committed NK precursor expresses the IL-2/IL-15 receptor common β subunit (CD122) correlating with the vital role played by IL-15 and its receptors in NK cell development (Koka et al., 2003; Lodolce et al., 2002; Rosmaraki et al., 2001). NK cells then mature in the BM via a linear developmental program that involves the sequential acquisition of NK1.1, the CD94-NKG2 and Ly-49 receptors, and the DX5 α2β1 integrin. These receptors have varied and critical roles in the regulation of NK cell effector function, and major recent advances have been made in understanding their function (Arase et al., 2002; Faure et al., 2003; Jamieson et al., 2002; Nakamura et al., 2000; Ogasawara et al., 2003; Raulet, 2003; Riteau et al., 2003; Seaman, 2000). Terminal maturation is accompanied by high expression of CD11b and CD43 together with optimal cytolytic function and IFN-γ production (Kim et al., 2002; Yokoyama et al., 2003). Mature NK cells are believed to be terminally differentiated and, once in the periphery, static in terms of proliferative capacity until challenge with pathogen. Radio-resistant BM cells producing IL-15 are critical for NK cell development and homeostasis (Kennedy et al., 2000; Ranson et al., 2003b). Other critical factors in the microenvironment include IL-7 and cell-cell contact between BM stromal cells and developing NK cells via lymphotoxin receptors and cell surface lymphotoxin, respectively (Colucci et al., 2003). Several transcription factors have been identified that are important for NK cell development. IRF-1 acts in a stem cell-extrinsic manner, explained by the defect in IL-15 production in IRF-1$^{-/-}$ BM (Ogasawara et al., 1998). Other factors such as IRF-2, ID2, Ets1, MEF, and PU.1 regulate NK development in a stem cell-intrinsic manner (Barton et al., 1998; Colucci et al., 2001; Lacorazza et al., 2002; Lohoff et al., 2000; Yokota et al., 1999), and mice lacking these transcription factors generally have developmental defects in multiple lymphoid lineages. However, the molecular mechanisms involved and the precise stage of development affected by loss of these factors remains unclear.

NKT cells possess properties of both T cells and NK cells in that they coexpress a T cell receptor (TCR) and a panoply of receptors of the NK lineage, including NK1.1, NKG2D, and members of the Ly-49 family (Bendelac et al., 1997; Ho et al., 2002). Recent progress has been made in identifying their developmental intermediates. The majority of NKT cells contain an invariant TCR rearrangement of Vα14 to Jα18 and are reactive to the glycolipid α-galactosylceramide (αGalCer) presented on CD1d, so-called Vα14 invariant (Vα14*i*) NKT cells (Kronenberg and Gapin, 2002). NKT cells develop from CD4$^+$CD8$^+$ double-positive (DP) Vα14-Jα18 TCR$^+$ thymic precursors which then upregulate CD44 expression; the terminal maturation step is accompanied by expression of NK1.1, Ly-49 receptors, and CD122 (Benlagha et al., 2002; Gadue and Stein, 2002; Gapin et al., 2001;

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Matsuda et al., 2002). Upon upregulation of CD122, Vα14*i* NKT cells proliferate in response to IL-15 (Matsuda et al., 2002; Ranson et al., 2003a) and migrate from the thymus to the periphery where they are most abundant in the spleen, liver, and BM. Mature CD1d-dependent Vα14*i* NKT cells exhibit a memory phenotype ($CD62L^{-}CD69^{+}CD44^{hi}$) and can be either $CD4^{+}$ single-positive (SP) or $CD4^{-}CD8^{-}$ double-negative (DN) cells. Developing Vα14*i* NKT cells express IL-4 but little IFN-γ, while mature cells express higher levels of IFN-γ and less IL-4 (Benlagha et al., 2002; Pellicci et al., 2002). IL-15, LTα/β, Ets1, and MEF affect NKT development, suggesting the conservation of regulatory genetic programs between NK and NKT cells (Kronenberg and Gapin, 2002; Lacorazza et al., 2002; Walunas et al., 2000).

The transcription factor T-bet (*T-box expressed in T* cells, also known as Tbx21) is a member of the T-box family of transcription factors that contain a conserved 200 amino acid DNA binding domain called the T-box. T-box proteins have been described in multiple species where they are critically involved in vertebrate developmental processes (Wilson and Conlon, 2002). T-bet is the only known T-box gene selectively expressed in the lymphoid system (Szabo et al., 2003). T-bet is rapidly expressed in Th1, but not Th2, cells via a combination of signaling from the TCR and the IFN-γR/STAT1 pathways, but not the IL-12/STAT4 pathway (Lighvani et al., 2001). Mice deficient in T-bet have established an important role for this transcription factor in Th1 immunity. $T\text{-bet}^{-/-}$ CD4 T cells have a severe defect in production of IFN-γ as do antigen-specific $T\text{-bet}^{-/-}$ CD8 and dendritic cells (Lugo-Villarino et al., 2003; Sullivan et al., 2003).

Here we report that mice lacking T-bet have stem cell-intrinsic defects in the maturation of the NK and Vα14*i* NKT cell lineages. Both cell lineages have blocks at a similar developmental stage leading to significantly decreased numbers in the periphery with a halt in terminal maturation. These results show a critical developmental role for T-bet in the innate immune system and suggest that there are common molecular pathways in operation during the final maturation stages of both NK and Vα14*i* NKT cells.

Results

Peripheral Deficiency and Impaired Maturation of NK and NKT Cells in the Absence of T-bet

T-bet-deficient mice backcrossed eight generations to the C57BL/6 background were utilized in these studies as this strain, unlike BALB/c, expresses the marker NK1.1. Furthermore, the C57BL/6 but not the BALB/c strain contains the *Chok* locus (Idris et al., 1999) containing the gene encoding the NK-activating receptor Ly-49D. To assess the role of T-bet on NK and NKT cell development, various tissues from naive WT (WT) and $T\text{-bet}^{-/-}$ mice were analyzed by flow cytometry using antibodies specific for NK1.1 and TCRβ to allow for visualization and differentiation of NK and NKT cells. Figure 1A shows that in spleen, liver, and peripheral blood there was a marked decrease in numbers of $NK1.1^{+}TCR\beta^{-}$ NK cells. Similar reductions in numbers of $T\text{-bet}^{-/-}$ NK cells were seen when tissues were stained with antibodies against DX5 and TCRβ, demonstrating that the observed deficiency could not be accounted for by a lack of expression of the NK1.1 marker (data not shown). In contrast to the deficiency seen in the periphery, there was a modest increase in numbers of $NK1.1^{+}TCR\beta^{-}$ NK cells in $T\text{-bet}^{-/-}$ BM. Further, examination of the thymus and BM in addition to the other peripheral organs revealed a nearly complete absence of $NK1.1^{+}TCR\beta^{+}$ NKT cells. Thus, the integrity of the NK and NKT compartments is markedly affected by the absence of T-bet.

To assess the developmental phenotype and activation status of $T\text{-bet}^{-/-}$ NK cells, splenocytes from WT and $T\text{-bet}^{-/-}$ mice were stained with antibodies against NK1.1, TCRβ, and a panel of markers known to reflect NK maturation. Figure 1B shows that $T\text{-bet}^{-/-}$ NK cells have profoundly reduced levels of CD43 (leukosialin) and reduced levels of CD11b (Mac1) and DX5 (α2β1 integrin), markers shown to be upregulated during NK development (Kim et al., 2002). Similarly, $T\text{-bet}^{-/-}$ NK cells showed increased expression of cKit and α_V integrin, markers that are expressed on developing NK cells but are downregulated upon maturation. Expression of CD45R (B220) on $T\text{-bet}^{-/-}$ NK cells is elevated and more homogeneous than on WT cells, a pattern that has been demonstrated previously on immature NK cells (Samson et al., 2003). Further, $T\text{-bet}^{-/-}$ NK cells expressed significantly higher levels of CD69 (Figure 1C), a marker shown to be upregulated upon NK activation (Ziegler et al., 1994), suggesting that NK cells are present in a hyperactivated state in naive $T\text{-bet}^{-/-}$ animals. Figure 1D shows that $T\text{-bet}^{-/-}$ NK cells express a normal repertoire of activating and inhibitory receptors, although a higher proportion of $T\text{-bet}^{-/-}$ compared to WT NK cells expressed both the activating receptor Ly-49D and the inhibitory receptor Ly-49G2.

NK development in the BM was also examined and revealed a similar halt in maturation as observed in the periphery as an increased number of immature $CD122^{+}NK1.1^{+}CD11b^{lo}$ cells were present. A decrease in the percentage of $CD122^{+}$ double-negative cells, the earliest known committed NK precursor (Kim et al., 2002) was also observed (Figure 1E). In contrast to the peripheral organs, NK cell numbers were increased rather than decreased (WT 0.37% versus $T\text{-bet}^{-/-}$ 0.56%), perhaps reflecting the increased turnover rate (see below). Both BM and peripheral $T\text{-bet}^{-/-}$ $NK1.1^{+}TCR\beta^{-}$ NK cells expressed normal levels of CD122 (Figure 1F) and proliferated normally in response to IL-2 and IL-15 in vitro (data not shown), although importantly this stimulation did not result in restoration of normal cell surface marker expression. Therefore, the decrease in peripheral NK cell numbers in $T\text{-bet}^{-/-}$ mice cannot be explained by their failure to respond to IL-15.

We conclude that there is a block in the terminal maturation of the NK lineage in $T\text{-bet}^{-/-}$ mice as demonstrated both by decreased numbers of peripheral NK cells and by perturbed expression of NK differentiation cell surface markers. These data are consistent with a

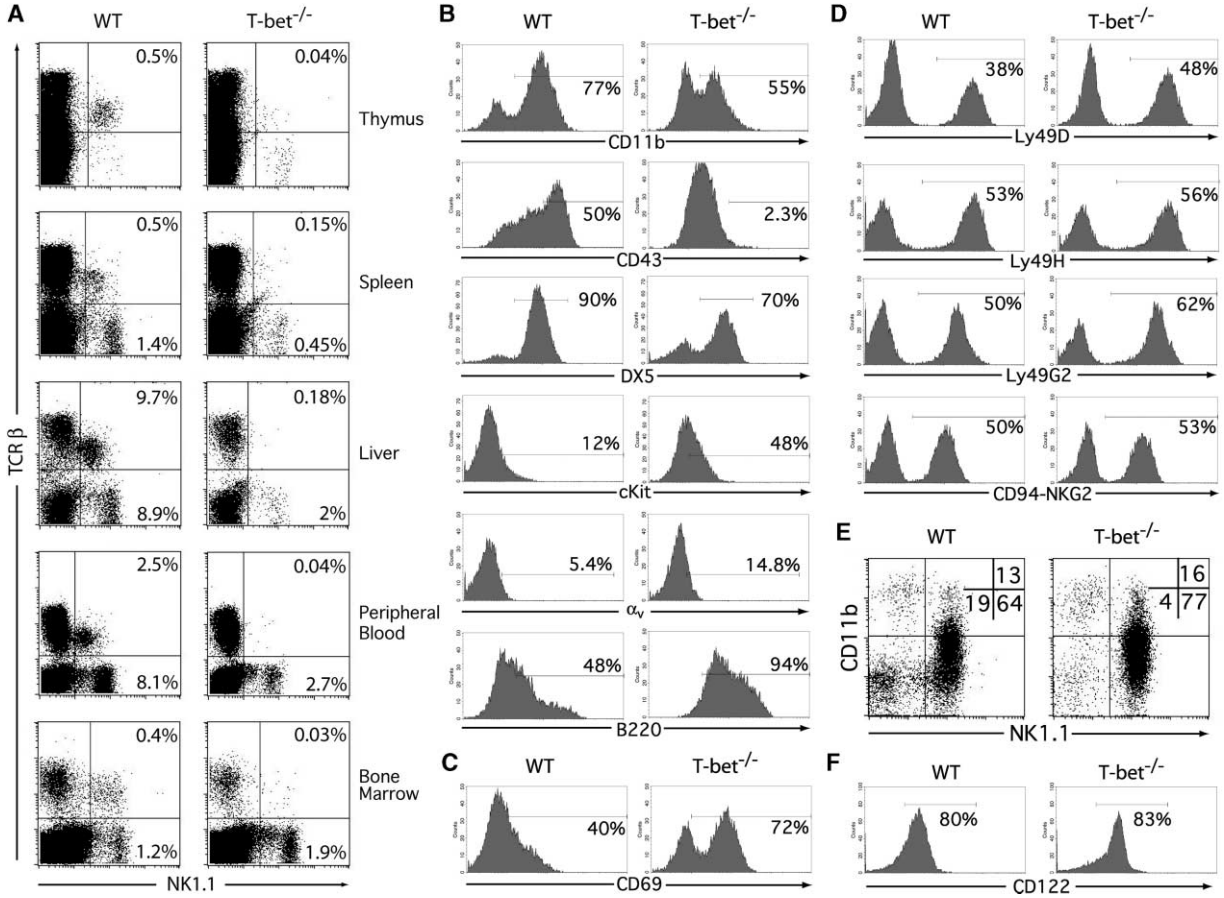


Figure 1. Peripheral Deficiency and Abnormal Development of NK and NKT Cells in T-bet$^{-/-}$ Mice

(A) Cells isolated from the indicated tissues of WT and T-bet$^{-/-}$ mice were stained for NK1.1 and TCRβ. The numbers of NK and NKT cells are shown as a percentage of total lymphocytes. Data are representative of at least five experiments.

(B) Splenocytes from WT and T-bet$^{-/-}$ mice were stained for a panel of antigens representing maturation markers. Gated NK1.1^{+}TCRβ^{-} cells are shown. Data are representative of at least three experiments.

(C) Splenocytes from WT and T-bet$^{-/-}$ mice were stained for the CD69 activation marker. Gated NK1.1^{+}TCRβ^{-} cells are shown. Data are representative of at least three experiments.

(D) Splenocytes from WT and T-bet$^{-/-}$ mice were stained with antibodies against NK activation and inhibitory receptors. Gated NK1.1^{+}TCRβ^{-} cells are shown. Data are representative of at least three experiments.

(E) BM NK developmental progression was determined by staining isolated marrow cells with CD122, TCRβ, NK1.1, and CD11b. Gated CD122^{+}TCRβ^{-} cells are shown, and the numbers represent the percentages of cells in the indicated quadrants. Data are representative of two experiments.

(F) Splenocytes from WT and T-bet$^{-/-}$ mice were stained for the CD122 receptor. Gated NK1.1^{+}TCRβ^{-} cells are shown. Data are representative of two experiments.

defect in the developmental maturation of NK cells and a perturbation of NK cell homeostasis in the absence of T-bet.

Increased Basal Turnover of T-bet$^{-/-}$ NK Cells Due to Increased Rates of Proliferation and Apoptosis

Immature developmental NK cell intermediates proliferate at specific stages, but mature cells have a very low basal proliferation rate (Kim et al., 2002). The increased numbers of NK precursors in the face of peripheral NK cell deficiency, coupled with evidence of hyperactivation, suggested alterations in cell turnover. We examined the rate of in vivo NK proliferation using BrdU incorporation. Figure 2A shows that only a very small percentage of WT NK1.1^{+}TCRβ^{-} NK cells incorporated BrdU, consistent with previous reports. In contrast, T-bet$^{-/-}$ NK cells incorporated BrdU at a significantly higher rate than WT cells. Staining for BrdU and total chromosomal DNA confirmed that these BrdU^{+} NK cells were traversing the cell cycle (data not shown). The disparity between the increased proliferation observed and the overall decreased numbers of peripheral NK cells suggested that increased cell death might also be occurring. It is possible that the peripheral BrdU^{+} NK cells in the T-bet$^{-/-}$ mice could also include new NK cells emerging from the BM during the duration of the BrdU pulse. However, staining of fresh splenocytes using the apoptotic marker Annexin V in conjunction with the dye 7-AAD to assess levels of cell death revealed significantly higher levels of early apoptotic (Annexin V^{+},

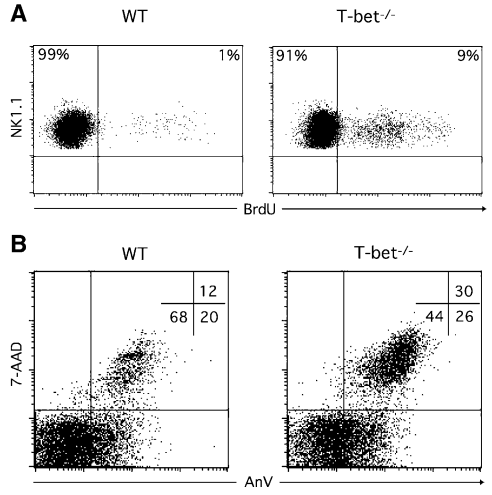


Figure 2. Increased NK Cell Basal Turnover and Higher NK Cell Apoptosis in T-bet$^{-/-}$ Mice

(A) WT and T-bet$^{-/-}$ mice were injected with BrdU. Three hours later, splenocytes were isolated and stained for NK1.1 and TCRβ. Cells were then stained for incorporated BrdU, and BrdU^{+} cells are shown as a percentage of the total NK (NK1.1^{+}TCRβ^{-}) population. Data are representative of two experiments.

(B) Fresh WT and T-bet$^{-/-}$ splenocytes were stained for NK1.1, CD3ε, Annexin V, and 7-AAD. Gated NK1.1^{+}CD3ε^{-} NK cells are shown, and the numbers represent the percentages of cells in each quadrant. Data are representative of two experiments.

7-AAD^{-}) and dead (Annexin V^{+}, 7-AAD^{+}) NK1.1^{+}TCRβ^{-} NK cells in T-bet$^{-/-}$ mice (Figure 2B). Thus, increased proliferation occurs in conjunction with significantly higher cell death.

Vα14*i* NKT Cells Exhibit a Severe Block in Development in the Absence of T-bet

Vα14*i* NKT cells comprise the vast majority of NK1.1^{+} TCRβ^{+} NKT cells in mice (Kronenberg and Gapin, 2002). However, NK1.1^{-} Vα14*i* NKT cells, identified with αGalCer-loaded CD1d tetramers, have also been reported in peripheral organs and are thought to represent immature recent thymic emigrants (Benlagha et al., 2002; Pellicci et al., 2002). The near absence of NK1.1^{+} TCRβ^{+} NKT cells in all the T-bet$^{-/-}$ organs studied (Figure 1A) prompted us to further examine this population using αGalCer-loaded CD1d tetramers that bind specifically to the invariant Vα14-Jα18 TCR (Matsuda et al., 2000). A striking reduction in the number of tetramer^{+} TCRβ^{+} NKT cells (Figure 3A) consistent with the observed decrease in NK1.1^{+}TCRβ^{+} cells was found in liver, spleen, and thymus of T-bet$^{-/-}$ mice. Further confirmation of a reduction in Vα14*i* NKT cells was obtained by quantitative real-time PCR using primers specific for the Vα14-Jα18 TCR rearrangement. Figure 3B shows a significant reduction in transcripts encoding this TCR rearrangement in thymic and splenic lymphocytes from T-bet$^{-/-}$ mice after normalization to Cα transcript levels. Since mice lacking CD1d have severe defects in NKT development (Chen et al., 1997; Mendiratta et al., 1997; Smiley et al., 1997), it was possible that the reduction in Vα14*i* NKT cells was secondary to defective expression of CD1d in T-bet$^{-/-}$ mice. However, examination of splenocytes from WT and T-bet$^{-/-}$ mice stained with antibodies against CD1d, CD19, and TCRβ revealed equivalent expression of CD1d on B and T cells (Figure 3C) ruling out CD1d deficiency as a cause of the Vα14*i* NKT defect.

Vα14*i* NKT cells developing in the thymus initially acquire the Vα14-Jα18 TCR rearrangement, then upregulate CD44, and finally NK1.1 as the terminal maturation step (Benlagha et al., 2002; Gapin et al., 2001; Matsuda et al., 2002). Analysis of tetramer^{+} thymocytes showed that while T-bet$^{-/-}$ cells mostly upregulate CD44, the vast majority failed to express NK1.1 in contrast to WT cells (Figure 3D). Enumeration of Vα14*i* NKT developmental intermediates in WT and T-bet$^{-/-}$ thymocytes revealed that the majority of tetramer^{+} NKT cells in T-bet$^{-/-}$ mice reside as CD44^{lo}NK1.1^{-} and CD44^{hi}NK1.1^{-} developmental intermediates, whereas the majority of WT tetramer^{+} cells are fully mature CD44^{hi}NK.1.1^{+} NKT cells (Figure 3E). The terminal maturation stages during Vα14*i* NKT development involve the upregulation of Ly-49 receptors (Gapin et al., 2001) and CD122 (Matsuda et al., 2002) in addition to NK1.1 (Benlagha et al., 2002), and NKT cells of adult mice have also been shown to express CD5 and CD69 (Benlagha et al., 2002; Matsuda et al., 2002). T-bet$^{-/-}$ tetramer^{+} thymocytes had low levels of expression of CD122 and failed to express Ly-49C, Ly-49G2 although they did express CD5 and CD69 (Figure 3F). Further, T-bet$^{-/-}$ αGalCer tetramer^{+} NKT cells were unable to respond to IL-15 stimulation in vitro, confirming functionally the low level of CD122 expression (see the Supplemental Data at http://www.immunity.com/cgi/content/full/20/4/477/DC1). Again, these results are consistent with a severe block in the development and maturation of the Vα14*i* NKT cell lineage in the absence of T-bet.

The Defects in NK and Vα14*i* NKT Development in T-bet$^{-/-}$ Mice Are Entirely Stem Cell Intrinsic and Not Microenvironment Dependent

The BM and thymic microenvironments regulate the development of NK and NKT cells both through the secretion of soluble cytokines that bind GM-CSF, IL-7, IL-15Rα, IL-2/15Rβ, and c-Kit receptors on developing cells and via cell surface-bound lymphotoxin (LT) that binds the LTβ receptor on stromal cells (Colucci et al., 2003; Kronenberg and Gapin, 2002; Lian and Kumar, 2002). We therefore investigated whether the absence of T-bet affected NK and NKT cell development via a stem cell-intrinsic mechanism, via a defective developmental microenvironment, or both.

BM reconstitution experiments were performed where lethally irradiated Ly5.1 congenic WT and T-bet$^{-/-}$ hosts were reconstituted with congenic WT or T-bet$^{-/-}$ BM to allow us to distinguish between WT and T-bet$^{-/-}$-derived peripheral lymphocytes. Figure 4A shows that WT BM reconstituted the NK cell compartment equally well both in WT hosts and in T-bet$^{-/-}$ hosts; in fact, WT BM gave better reconstitution of T-bet$^{-/-}$ hosts than of WT hosts. In contrast, T-bet$^{-/-}$ BM reconstituted the NK compartment poorly both in WT and T-bet$^{-/-}$ hosts (Figure 4A). Similarly, the Vα14*i* NKT cell compartment, as assessed by staining with αGalCer/CD1d tetramer and TCRβ (Figure 4B) or for NK1.1 and TCRβ (Figure

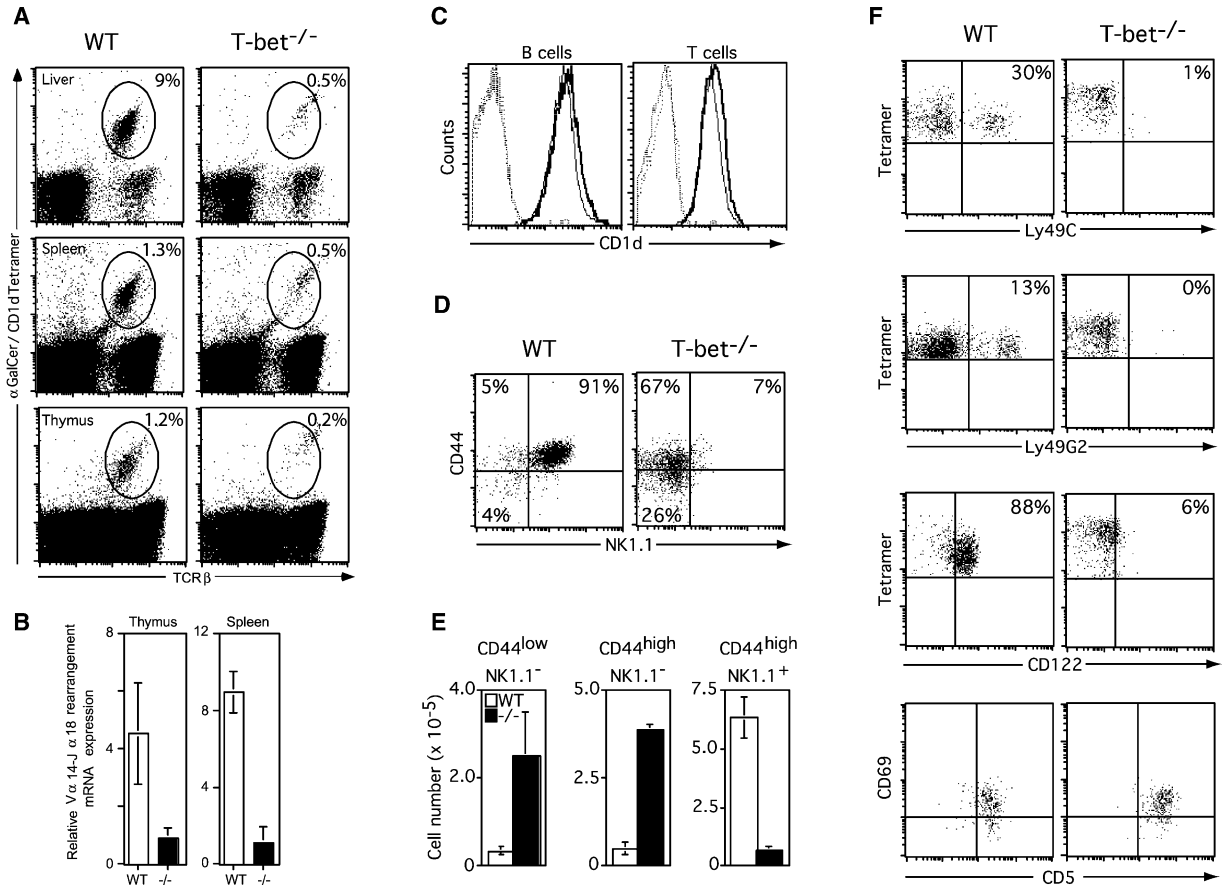


Figure 3. Analysis of Vα14*i* NKT Cell Deficiency and the Developmental Intermediates of Vα14*i* NKT Cells in T-bet$^{-/-}$ Mice

(A) Reduced percentage of αGalCer/CD1d tetramer$^+$ TCRβ$^+$ cells in the liver, spleen, and thymus of T-bet$^{-/-}$ compared to WT control mice. The specificity of αGalCer/CD1d tetramer staining was demonstrated by the absence of staining in the organs of Jα18$^{-/-}$ mice as well as the absence of staining with CD1d tetramer that was not loaded with αGalCer (data not shown). Data are representative of four experiments.

(B) Amounts of Vα14-Jα18 transcripts in the thymus and the spleen of WT and T-bet$^{-/-}$ mice were quantified by quantitative real-time PCR. Normalization of the samples was relative to the quantity of Cα transcripts. Data are representative of two experiments.

(C) Analysis of CD1d expression in T-bet$^{-/-}$ mice. Splenocytes from WT and T-bet$^{-/-}$ mice were stained with anti-CD1d mAb or isotype control and analyzed by flow cytometry. Expression levels of CD1d on B cells (CD19$^+$) or T cells (TCRβ$^+$) are shown.

(D) Analysis of expression of CD44 and NK1.1 on tetramer$^+$ thymocytes. Gated αGalCer/CD1d tetramer$^+$ TCRβ$^+$ cells are shown, along with percentages of cells in the indicated quadrants. Data are representative of three experiments.

(E) Enumeration of αGalCer/CD1d tetramer$^+$ TCRβ$^+$ thymocytes in wild-type and T-bet$^{-/-}$ mice based on their CD44 and NK1.1 expression pattern.

(F) Analysis of expression of LY-49C/I, Ly-49G2, CD122, CD5, and CD69 on tetramer$^+$ thymocytes from wild-type and T-bet$^{-/-}$ mice. Data are representative of three experiments.

4A), was reconstituted by WT BM equally well in WT and T-bet$^{-/-}$ mice. However, T-bet$^{-/-}$ BM reconstituted the Vα14*i* NKT compartment poorly in both host genotypes (Figure 4B).

Thus, our results argued strongly for a purely stem cell-dependent mechanism to explain the NK and NKT cell defects. However, the formal possibility remained that other cell types in the BM (e.g., macrophages, stromal cells, and dendritic cells that produce immunoregulatory cytokines and cell-cell contact essential for NK and Vα14*i* NKT cell development) were also defective due to the absence of T-bet and were being transferred along with the stem cells into the lethally irradiated host. While BM stromal cells are radio resistant, the other host-derived cell types will die and so cannot contribute growth factors to the developing NK and Vα14*i* NKT cells. To address this issue, a competitive reconstitution experiment was performed where equal numbers of congenic WT and T-bet$^{-/-}$ BM cells were mixed together and injected into lethally irradiated Rag2$^{-/-}\gamma_c^{-/-}$ hosts. These hosts lack T, B, NK, or Vα14*i* NKT cells, so all of these lymphocytes must be reconstituted from donor marrow. The mixing of WT and T-bet$^{-/-}$ marrow ensured WT cell lineages would be present during the maturation of T-bet$^{-/-}$ NK and Vα14*i* NKT cell progenitors. Analysis of these mice revealed that the contribution of T-bet$^{-/-}$ BM to the NK and NKT cell compartments was again significantly lower than that of WT BM (Figures 4C and 4D). This result shows conclusively that the observed defects in NK and Vα14*i* NKT development in the absence of T-bet are entirely stem cell intrinsic, and furthermore that the BM and thymic microenvironments of

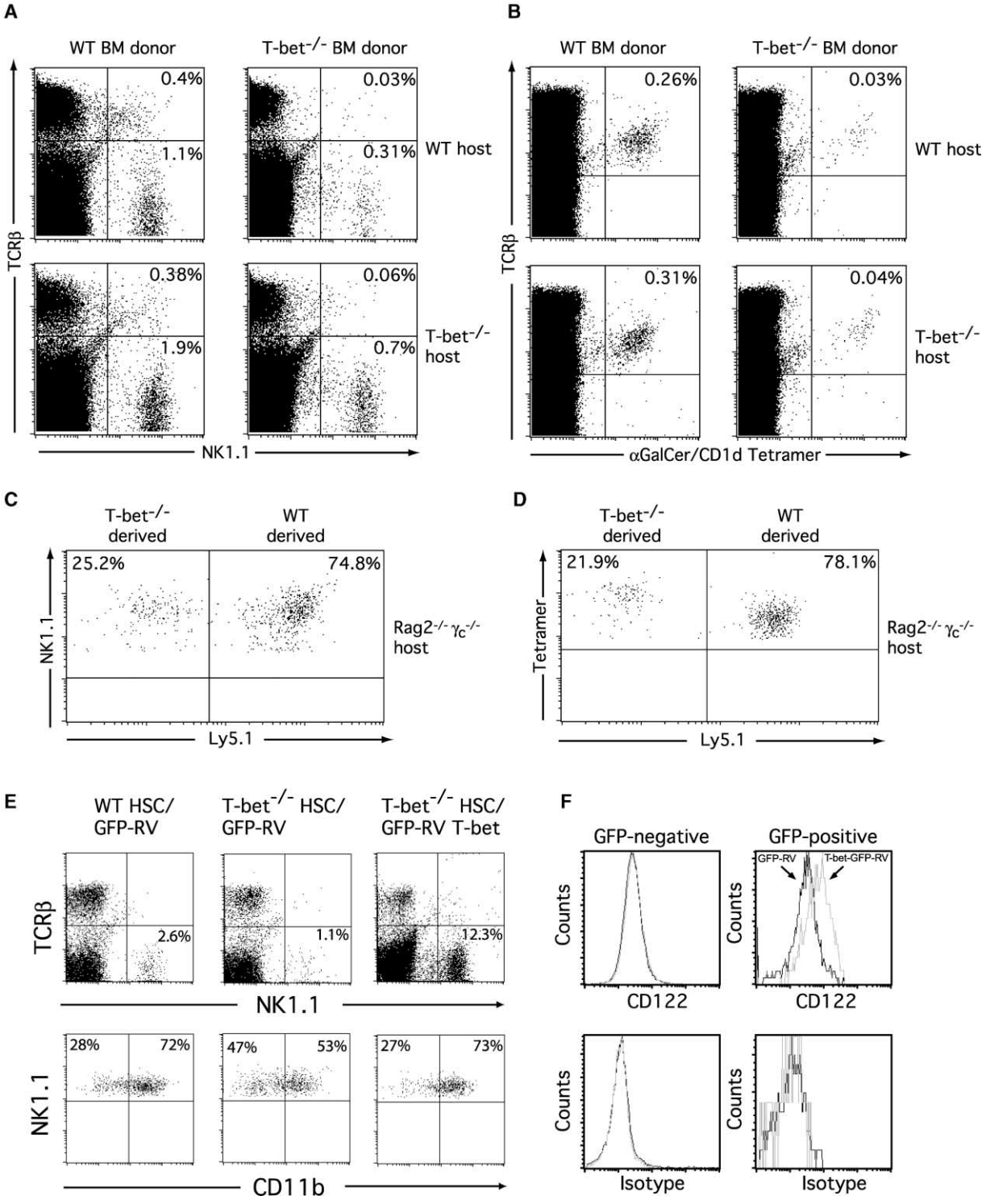


Figure 4. The Deficiency of NK and NKT Cell Development in T-bet$^{-/-}$ Mice Is Stem Cell Intrinsic and Not Microenvironment Dependent

(A) WT and T-bet$^{-/-}$ lethally irradiated hosts were reconstituted with Ly5.1 congenic wild-type or T-bet$^{-/-}$ BM cells. Six weeks later, host splenocytes were examined by staining with NK1.1, TCRβ, and Ly5.1. Cells are gated on Ly5.1^{+} cells if host animals received WT congenic BM, or Ly5.1^{-} cells if hosts received T-bet$^{-/-}$ BM. The percentages of NK and NKT cells in these gates are shown. Data are representative of two experiments.

(B) Thymocytes of mice reconstituted as in (A) were examined by staining with αGalCer/CD1d tetramer, TCRβ, and Ly5.1. Cells are gated on Ly5.1^{+} cells if host animals received WT congenic BM, or Ly5.1^{-} cells if hosts received T-bet$^{-/-}$ BM cells. The percentages of NK and NKT cells in these gates are shown. Data are representative of two experiments.

(C) Rag2$^{-/-}\gamma_c^{-/-}$ lethally irradiated hosts were reconstituted with a 1:1 ratio of WT Ly5.1 congenic and T-bet$^{-/-}$ BM cells. Six weeks later, host splenocytes were examined by staining with NK1.1, TCRβ, and Ly5.1. Gated NK1.1^{+}TCRβ^{-} cells are shown, and the percentages of

T-bet$^{-/-}$ mice are sufficient to support NK and Vα14*i* NKT cell development when WT stem cells are provided.

IFN-γ might affect NK development and previous work has shown that T-bet controls IFN-γ production in various cell lineages (Lugo-Villarino et al., 2003; Sullivan et al., 2003; Szabo et al., 2002). However, mice genetically deficient in IFN-γ, IFN-γR, and STAT1 did not display any defects in NK and Vα14*i* NKT cell development or in cell numbers as measured by staining with NK1.1 and αGalCer/CD1d tetramer (data not shown and Supplemental Data) consistent with earlier reports (Dalton et al., 1993). Thus, the observed defects in the T-bet$^{-/-}$ mice cannot be explained by a decrease in systemic IFN-γ levels. We further examined the expression of IL-15 from T-bet$^{-/-}$ BM cells. Cells from WT and T-bet$^{-/-}$ mice were stimulated for various times with LPS and IFN-γ, and the resulting RNA was examined by Northern blot and real-time RT-PCR. IL-15 was induced with equal kinetics and quantity (Supplemental Data). Expression of IL-27, a cytokine shown to induce T-bet expression in CD4$^+$ T cells (Takeda et al., 2003), was also found to be normal in WT and T-bet$^{-/-}$ dendritic cells (Supplemental Data). Furthermore, expression of LT and its receptor were found to be normal in T-bet$^{-/-}$ mice (data not shown).

To definitively establish that the function of T-bet in controlling NK maturation was stem cell intrinsic, we transduced T-bet$^{-/-}$ hematopoietic stem cells (HSCs) with retroviruses expressing T-bet to rescue the phenotype. This experiment also yielded information about the effects of T-bet overexpression on NK and Vα14*i* NKT development complementary to what we had observed in the absence of T-bet. Figure 4E shows that retroviral overexpression of T-bet in T-bet$^{-/-}$ HSCs results in a marked increase in splenic NK1.1$^+$TCRβ^- NK cells compared with WT and with T-bet$^{-/-}$ cells transduced with empty retrovirus. Strikingly, overexpression of T-bet in T-bet$^{-/-}$ HSCs also results in the restoration of NK cell surface CD11b integrin expression, a marker of NK maturation, to levels comparable to that of WT NK cells. Therefore, T-bet overexpression results both in an increase in peripheral NK cell numbers and the restoration of phenotypic cell surface maturity.

Since we could not detect reproducible restoration of the Vα14*i* NKT cell compartment with this approach due to low expression of GFP in the thymus, we expressed T-bet by retroviral transduction of thymic NKT cells rather than HSCs. Immature Vα14*i* NKT cells have been reported to proliferate in response to IL-7 (Gadue and Stein, 2002) and while T-bet$^{-/-}$ Vα14*i* NKT cells were unable to respond to IL-15 due to the low expression levels of CD122, they proliferated to exogenous addition of IL-7 (data not shown). T-bet$^{-/-}$ thymi were depleted of CD8 cells and placed in culture with IL-7 (10 ng/ml) for 4 days, and cells then infected with GFP-RV or T-bet-GFP-RV and replated in culture with IL-7. Four days later, the expression of CD122 was monitored on GFP-positive and GFP-negative tetramer$^+$ cells. As shown in Figure 4F, the expression of CD122, low on developing NKT cells, was upregulated in the T-bet but not the control transduced cell cultures, as the cells developed. These experiments provide very strong evidence for a critical stem cell-intrinsic role for T-bet in the generation and terminal maturation of NK and Vα14*i* NKT cells. They further reveal a role for T-bet in controlling the homeostasis of NK cells in peripheral lymphoid organs.

T-bet Is Developmentally Regulated during NK and Vα14*i* NKT Cell Development and Is Induced by Multiple Stimuli

The defects observed above suggested that T-bet acted at defined stages of NK and Vα14*i* NKT cell differentiation, and it was of interest therefore to assess the regulation of T-bet expression during the development of these lineages. Developmental subsets were purified from BM and spleen and immediately analyzed for their expression of T-bet mRNA. Figure 5A shows that while CD122$^+$ NK1.1$^-$ NK precursor (NKP) cells express low levels of T-bet, NK1.1$^+$TCRβ^- NK cells from the BM and spleen express higher levels of T-bet mRNA as determined by real-time RT-PCR. Thus, T-bet expression in NK cells correlates with the progression of maturation. Levels of GATA3 also increase upon NK cell maturation, consistent with an earlier report showing expression of GATA3 transcripts in immature NK cells and more recent work demonstrating an important function for GATA-3 in NK cells (Rosmaraki et al., 2001; Samson et al., 2003).

To enable a similar analysis for Vα14*i* NKT cells, populations of thymic tetramer$^+$CD44^{lo}NK1.1$^-$, tetramer$^+$CD44^{hi} NK1.1$^-$ and tetramer$^+$CD44^{hi}NK1.1$^+$ cells were purified which represent successive stages in Vα14*i* cell development. Analysis of T-bet mRNA levels by real-time RT-PCR shows that T-bet expression increases upon maturation, while GATA3 levels stay relatively constant as normalized to housekeeping genes (Figure 5B). These results are consistent with a role for T-bet in the terminal

Ly5.1$^+$ (WT congenic-derived NK cells) to Ly5.1$^-$ (T-bet$^{-/-}$-derived NK cells) are shown. Data are representative of two experiments.

(D) Rag2$^{-/-}\gamma_c^{-/-}$ lethally irradiated hosts were reconstituted as in (C). Six weeks later, host thymocytes were examined by staining with αGalCer/CD1d tetramer, TCRβ, and Ly5.1. Gated tetramer$^+$TCRβ^+ cells are shown, and the percentages of Ly5.1$^+$ (WT congenic-derived NKT cells) to Ly5.1$^-$ (T-bet$^{-/-}$-derived NKT cells) are shown. Data are representative of two experiments.

(E) In vivo retroviral expression of T-bet in T-bet$^{-/-}$ HSCs results in expansion of the NK1.1$^+$ TCRβ^- NK cell compartment and the restoration of wild-type NK cell integrin expression levels. Lethally irradiated Rag2$^{-/-}\gamma_c^{-/-}$ hosts were reconstituted with WT or T-bet$^{-/-}$ HSCs infected with T-bet GFP-RV retrovirus or empty control retrovirus. Eight weeks later splenocytes from the reconstituted hosts were stained with antibodies against NK1.1 and TCRβ, and GFP$^+$ (virus-positive) cells are shown. The percentages of cells in the NK1.1$^+$ TCRβ^- quadrants are shown. Splenocytes from the reconstituted Rag2$^{-/-}\gamma_c^{-/-}$ hosts were also stained with antibodies against NK1.1, TCRβ, and CD11b. The plots show GFP$^+$NK1.1$^+$TCRβ^- NK cells. WT and T-bet$^{-/-}$ HSCs transduced with the empty retrovirus vector are shown as controls. Data are representative of two experiments.

(F) In vitro retroviral expression of T-bet in T-bet$^{-/-}$ thymocytes results in restoration of expression of CD122 on αGalCer tetramer$^+$ T-bet$^{-/-}$ NKT cells. Thymocytes from T-bet$^{-/-}$ mice were depleted of CD8$^+$ cells and cultured with IL-7 for 4 days. The cells were then infected with GFP-RV or GFP-RV T-bet retroviruses. After a further 4 days, the expression of CD122 was examined on GFP$^+$ αGalCer tetramer$^+$ cells using flow cytometry. Isotype controls show specificity of staining of αCD122 antibody. Data are representative of two experiments.

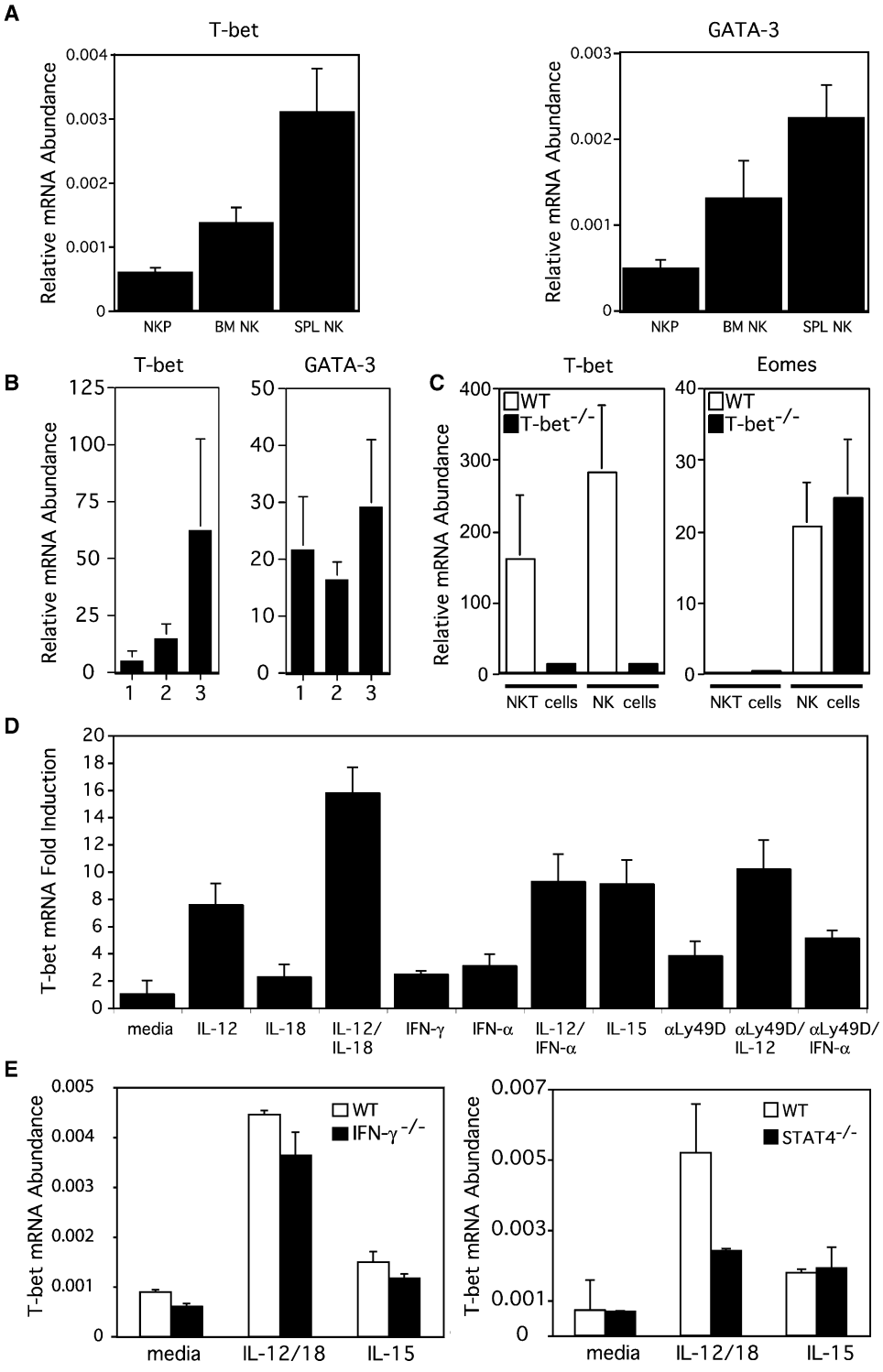


Figure 5. Signals Inducing T-bet Expression in NK Cells

(A) Upregulation of T-bet during the development of NK cells. BM-derived CD122^{+}NK1.1^{-}TCRβ^{-} NKP cells, BM-derived NK1.1^{+}TCRβ^{-} cells, and splenic NK1.1^{+}TCRβ^{-} cells were sorted from wild-type mice. Total RNA was prepared from these cells, and quantitative real-time RT-PCR analysis was performed using primers and probes specific for T-bet and GATA-3. The amounts of T-bet and GATA-3 transcripts were determined by quantitative real-time PCR with normalization to the amount of β-actin for each sample.

(B) Upregulation of T-bet during the development of Vα14*i* NKT cells. Fresh developmental intermediates (CD44^{low}NK1.1^{-} [1], CD44^{high}NK1.1^{-} [2], and CD44^{high}NK1.1^{+} [3]) of thymic CD1d tetramer^{+} Vα14*i* NKT cells from C57BL/6 mice were sorted and total RNA purified. The amounts of T-bet and GATA-3 transcripts in each population were determined by quantitative real-time RT-PCR with normalization to the amount of HPRT in each sample.

(C) Differential expression of Eomesodermin (Eomes) by NK and Vα14*i* NKT cells. Fresh NK1.1^{+}TCRβ^{-} NK cells and αGalCer/CD1d tetramer^{+}

maturation of Vα14*i* NKT cells, and correlate with previous observations that Vα14*i* NKT cells produce IFN-γ, but less IL-4, upon maturation (Benlagha et al., 2002; Stetson et al., 2003). Indeed, this was confirmed by our own observations that levels of IFN-γ transcripts increase upon cell maturation while IL-4 levels decrease (data not shown).

Another T-box transcription factor, Eomesodermin (Eomes), has recently been shown to be expressed in IL-2-expanded NK cells and $CD8^+$ T cells, but not in $CD4^+$ T cells, and has important functions in promoting cytotoxicity and IFN-γ secretion (Pearce et al., 2003). It was therefore of interest to examine the expression of this factor in Vα14*i* NKT cells and primary NK cells ex vivo by real-time RT-PCR. Figure 5C shows that while T-bet is expressed in both NK and Vα14*i* NKT cell lineages, Eomes is expressed only in the NK cell lineage. The lack of Eomes expression in Vα14*i* NKT cells provides an intriguing parallel to its lack of expression in $CD4^+$ T cells. Importantly, Eomes expression by NK cells is unaffected by the absence of T-bet, and this has obvious implications for a potential compensatory role of Eomes in T-bet$^{-/-}$ NK cell effector function and development.

We investigated the identity of the upstream signals required to induce T-bet in NK cells. WT NK cells were purified and stimulated with various combinations of cytokines and an agonistic antibody against the activating receptor Ly-49D, stimuli that have been implicated in NK activation and/or proliferation (Lauwerys et al., 1999; Ortaldo et al., 2001; Ortaldo and Young, 2003). Quantitative real-time RT-PCR analysis was performed to assess T-bet mRNA levels. Figure 5D shows that the cytokines IL-12 and IL-15 induced T-bet expression strongly. Other cytokines such as IL-18, IFN-α, and IFN-γ did not cause significant T-bet induction. In addition, ligation and crosslinking of the Ly-49D receptor modestly induced T-bet. In particular, IL-12 together with IL-18, which has been shown to be a powerful inducer of IFN-γ production by NK cells (Lauwerys et al., 1999), was the most potent inducer of T-bet expression observed. T-bet induction was also augmented when the Ly-49D receptor was stimulated in combination with IL-12 or IFN-α as compared with ligation of the receptor or cytokine alone. It is significant that IL-15 alone strongly induced T-bet expression as this links the proposed function of T-bet in NK cell maturation with the known critical role for IL-15 in NK cell development. In addition, we have observed a significant increase in T-bet transcript levels in thymic Vα14*i* NKT cells expanded with IL-15 (data not shown), indicating that the IL-15-T-bet pathway might be operative in this cell lineage as well.

Previous studies have shown that IFN-γ is the major inducer of T-bet in various cell lineages (Lighvani et al., 2001). Surprisingly, however, regulation of T-bet expression in NK cells is IFN-γ independent, as IFN-$\gamma^{-/-}$ NK cells have no impairment in T-bet expression (Figure 5E). Consistent with the potent induction of T-bet by IL-12 and IL-18, STAT4$^{-/-}$ NK cells have a significant defect in T-bet induction upon treatment with these cytokines but not with IL-15 (Figure 5E). Therefore, in contrast to T cells or dendritic cells (Afkarian et al., 2002; Lighvani et al., 2001; Lugo-Villarino et al., 2003), the STAT4 signaling pathway is important for T-bet induction in NK cells.

Identification of Putative T-bet Target Genes in NK Cells

Currently, there are few known target genes for T-bet. T-bet was cloned as a factor that transactivated IL-2 in a yeast one-hybrid screen and was subsequently shown to be an important regulator of IFN-γ and the IL-12Rβ chain (Afkarian et al., 2002; Szabo et al., 2000, 2002). In an effort to uncover novel target genes of T-bet in NK cells, we utilized an approach that couples chromatin immunopreciptation with CpG island microarray analysis (Weinmann and Farnham, 2002; Weinmann et al., 2002). This approach allows for the rapid analysis of promoters that are directly and specifically bound by a defined transcription factor. Initially, the human NK cell line YT was utilized, and chromatin from these cells was immunoprecipitated using an antibody that is reactive to both human and mouse T-bet. After fluorochrome conjugation, the chromatin was used to probe a microarray containing 7776 CpG islands. Array clones were identified for which the signal obtained with the T-bet-immunoprecipitated chromatin was at least 2-fold higher than the signal from a no-antibody control chromatin sample. These clones were subsequently verified by standard immunoprecipitation (ChIP) experiments using an antibody directed against T-bet, together with a no-antibody control. Further verification was carried out by ChIP using murine chromatin isolated from IL-2-expanded lymphokine-activated killer (LAK) cells derived from WT and T-bet$^{-/-}$ splenic NK cells. For this analysis, primer sets were prepared that spanned ~300 bp of the promoter upstream of the transcription start site of its corresponding gene. Due to the average size of the chromatin after sonication, T-bet binding could be detected in a region within 1 kb of the primer pair. In addition, hypothesized targets (not from the array) were confirmed to be direct targets using the standard ChIP assay. Real-time RT-PCR of putative target genes was then performed to confirm perturbed expression in the absence of T-bet.

Vα14*i* NKT cells were sorted from spleens of WT and T-bet$^{-/-}$ mice and total RNA purified. The amounts of T-bet and Eomes transcripts in each population were determined by quantitative real-time RT-PCR with normalization to the amount of HPRT in each sample.

(D) T-bet expression by NK cells is induced by multiple different stimuli. WT splenic NK cells were purified and stimulated for 12 hr with media alone or the indicated cytokines and/or antibody against the Ly-49D receptor. RNA was prepared and real-time RT-PCR performed using primers and probes specific for T-bet and β-actin. The levels of T-bet were normalized to β-actin, and the fold induction of T-bet as compared with media alone for each stimulation is shown. Data are representative of three experiments.

(E) T-bet expression by NK cells is induced independently of IFN-γ but partially requires STAT4. Splenic NK cells were purified from wild-type, IFN-$\gamma^{-/-}$, and STAT4$^{-/-}$ mice, and equal numbers were stimulated for 12 hr as indicated. RNA was prepared from the cells and real-time RT-PCR performed using primers and probes against T-bet and β-actin. The amounts of T-bet transcripts in each population were determined by quantitative real-time PCR with normalization to the amount of β-actin for each sample. Data are representative of three experiments.

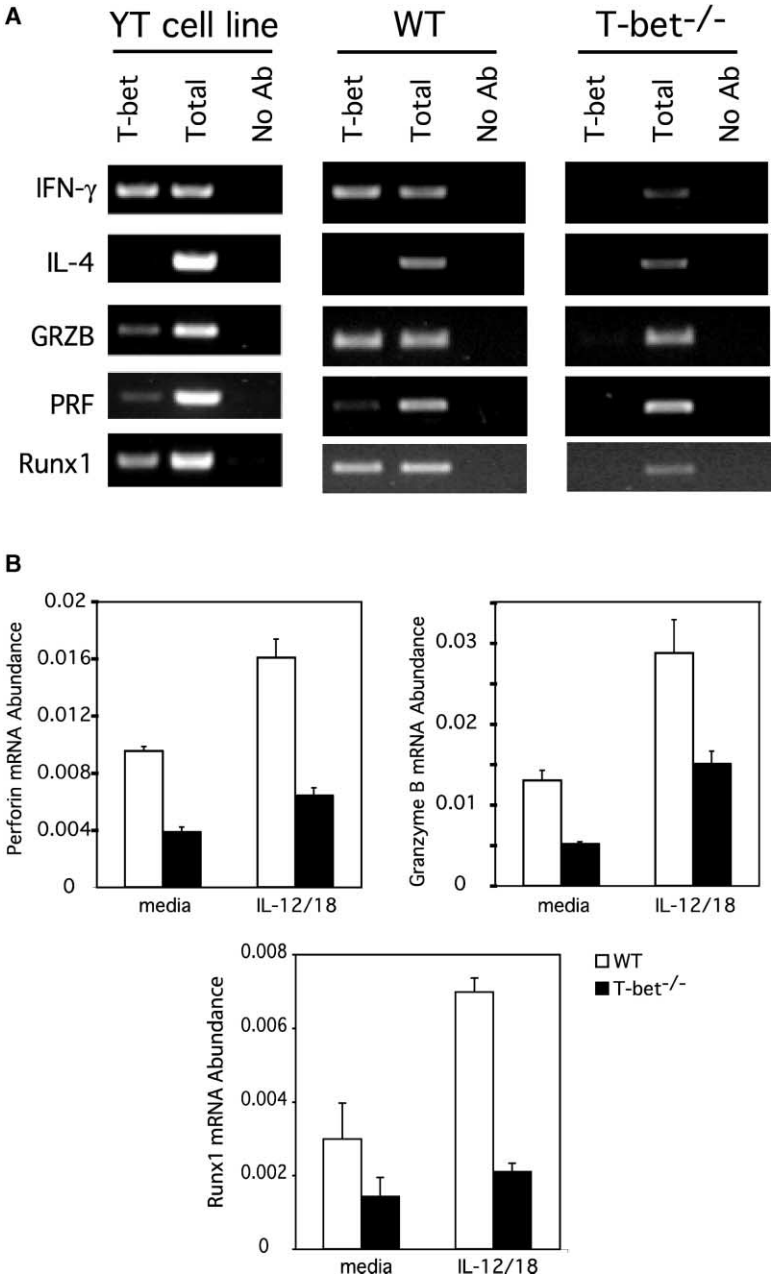


Figure 6. Putative T-bet Target Genes in NK Cells Identified by Chromatin Immunoprecipitation and Expression Level Analysis

(A) T-bet binds to the promoters of granzyme B (GRZB), perforin (PRF), and Runx1, as determined by ChIP. Nuclear extracts from the human NK cell line YT, and IL-2-expanded, IL-12/IL-18-activated murine LAK cells were incubated with antibodies against T-bet, and chromatin was immunoprecipitated. Primers specific to the promoter regions of these genes were used to detect enrichment of these promoters in the DNA from the immunoprecipitated samples. IFN-γ and IL-4 are shown as positive and negative controls, respectively. A standardized aliquot of the input chromatin is also shown (total) together with a no-antibody control. In addition, no T-bet-specific signal was detected in chromatin from T-bet$^{-/-}$ LAK cells. Data are representative of two experiments.

(B) Real-time RT-PCR analysis of perforin, granzyme B, and Runx1 expression in murine NK cells. Purified NK cells from wild-type and T-bet$^{-/-}$ mice were stimulated with media alone or IL-12 and IL-18 for 6 hr. RNA was then prepared from these cells and quantitative real-time PCR performed using primers and probes specific for these genes. The mRNA levels are normalized to β-actin levels. Data are representative of two experiments.

Figure 6A shows the ChIP confirmation of three putative T-bet targets, granzyme B, perforin and Runx1. Other target genes whose functional significance is unclear were also identified with the ChIP-CpG approach. IFN-γ is shown as a positive control and IL-4 as a negative control in addition to a no-antibody control. T-bet bound to the promoters of these three genes in the human YT cell line and WT murine LAK cells, but there was no detectable binding in the T-bet$^{-/-}$ control LAK chromatin preparation. In addition, the binding of T-bet to the mouse homologs confirmed specificity of binding in human chromatin. It is noteworthy that Runx1 has been shown to have specific promoters for T/B cells (distal) and NK/myeloid cells (proximal) (Telfer and Rothenberg, 2001) and that the ChIP analysis confirmed that T-bet binds to the NK-specific proximal promoter. Figure 6B shows the expression analysis of these genes by real-time RT-PCR. For these three genes there was a modest but consistent (2- to 3-fold) decrease in transcript levels in both resting cells and in cells stimulated with IL-12 and IL-18 (the stimulus shown to be the most potent inducer of T-bet in NK cells). Therefore, we assert that perforin, granzyme B, and Runx1 are T-bet target genes on the basis of direct binding of T-bet to their promoters together with decreased expression in T-bet$^{-/-}$ NK cells under both resting and stimulated conditions.

Effector Functions of T-bet$^{-/-}$ NK and Vα14*i* NKT Cells

The effector functions of NK cells during pathogen or tumor challenge include rapid secretion of large quantities of IFN-γ together with perforin- and granzyme-

dependent cytolysis of target cells (Biron et al., 1999; Kagi et al., 1994; Pardo et al., 2002). Mature Vα14*i* NKT cells produce IFN-γ upon engagement of their restricted TCR with CD1d-presented αGalCer. Figure 7A shows, as expected, that NK cells stimulated with IL-12 and IL-18, but not resting NK cells, rapidly secrete large quantities of IFN-γ. However, this early burst of IFN-γ was not impaired in the absence of T-bet. Interestingly, after 24 hr of stimulation, T-bet$^{-/-}$ cells produced significantly lower levels of IFN-γ, an observation consistent with the higher rate of cell death as determined by Annexin V staining (Figure 2 and data not shown). Similar observations were made after stimulation of NK cells with IL-12, PMA and ionomycin, or an antibody against Ly-49D (data not shown). Thus, the early and rapid secretion of IFN-γ is T-bet independent while the maintenance of IFN-γ production is impaired in the absence of T-bet, consistent with our earlier work (Szabo et al., 2002). Next, IL-12/IL-18-activated splenic NK cells from WT and T-bet$^{-/-}$ mice were examined for their ability to lyse the prototypic NK target cell YAC1 using a standard ^{51}Cr release assay. Figure 7B shows that, cell for cell, there was a modest but statistically significant decrease in the ability of T-bet$^{-/-}$ cells to lyse YAC1 target cells.

T-bet$^{-/-}$ Vα14*i* NKT cells were also tested for their ability to secrete IFN-γ by administering αGalCer in vivo, and examining cells ex vivo 75 min later. Figure 7C shows that while WT αGalCer tetramer^{+} cells produced IFN-γ rapidly after stimulation, T-bet$^{-/-}$ cells failed to make any detectable IFN-γ (staining was comparable to the isotype control antibody). Furthermore, splenocytes from αGalCer-treated mice cultured in vitro for 3 days produced undetectable amounts of IFN-γ (data not shown), consistent with the failure to detect IFN-γ by intracellular cytokine staining. Therefore, T-bet$^{-/-}$ Vα14*i* NKT cells express little, if any, IFN-γ, and this observation is consistent with the observed block in maturation to the stage where this cytokine is normally produced.

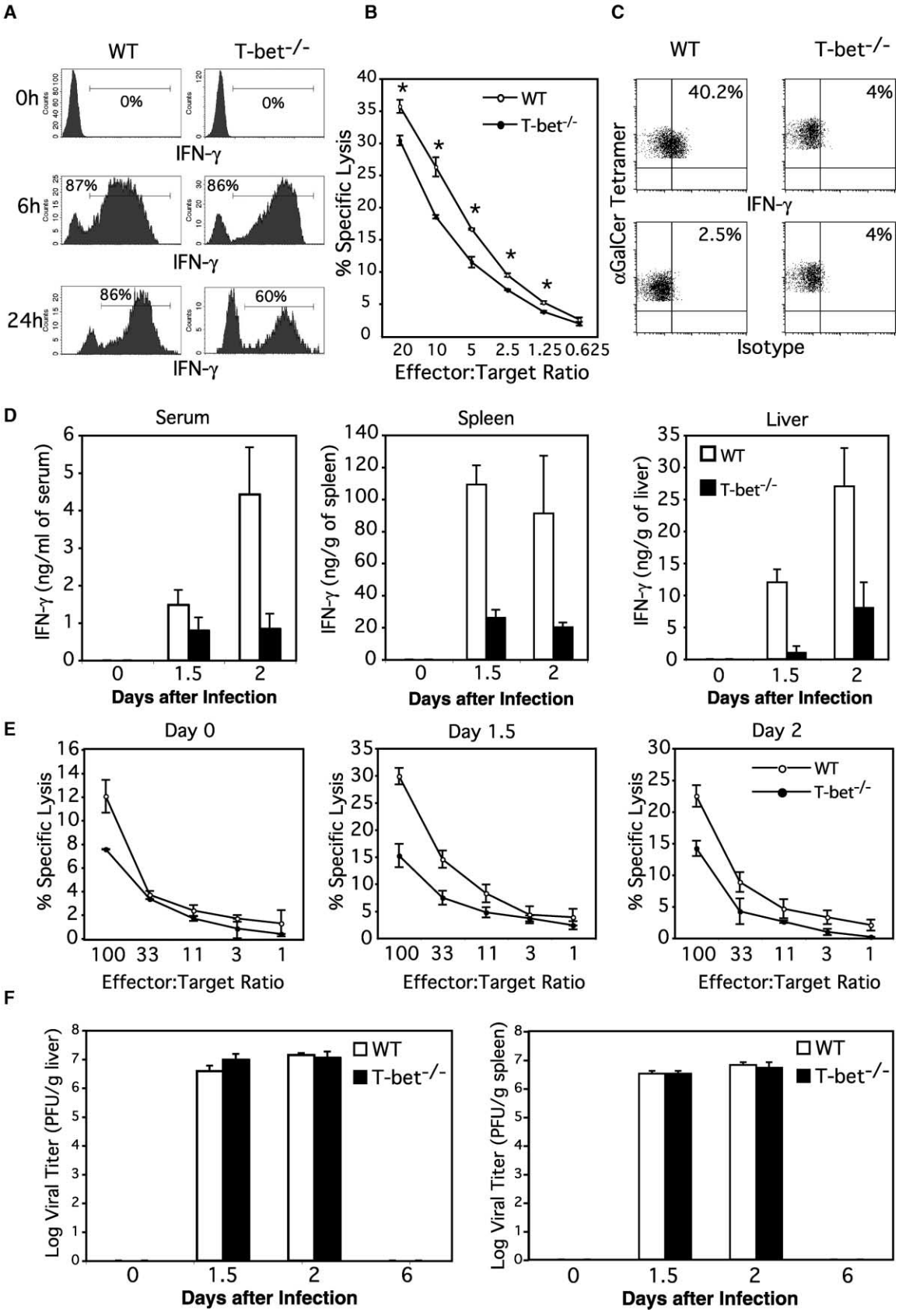
Finally, WT and T-bet$^{-/-}$ mice were infected with murine cytomegalovirus (MCMV) to assess the function of NK cells to an in vivo pathogen. MCMV clearance has been shown to be functionally dependent on NK cells (Biron et al., 1999). Figure 7D shows that levels of IFN-γ in the serum, spleen, and liver of infected mice were significantly decreased at both days 1.5 and 2 postinfection, consistent with both the decreased numbers of NK and Vα14*i* NKT cells and the decreased ability of these cells to secrete IFN-γ. At these early time points, the immune response is primarily limited to NK and not T cells. Furthermore, splenic cytolytic capacity was diminished in T-bet$^{-/-}$ mice both in uninfected and infected mice (Figure 7E), again consistent with both the decreased numbers and the diminished killing capacity of T-bet$^{-/-}$ NK cells. However, there was no difference in MCMV viral titers in the liver and spleen of T-bet$^{-/-}$ mice compared with WT (Figure 7F), indicating that NK cells are present at sufficient functional levels to control infection.

Discussion

Although T-bet was initially appreciated as a key transcription factor in Th1 lineage specification, it is becoming increasingly apparent that it also functions in other cell types in both innate and adaptive immunity. T-bet controls IgG2a gene transcription in B cells as well as effector functions and IFN-γ gene transcription in CD8^{+} T cells and dendritic cells (Lugo-Villarino et al., 2003; Peng et al., 2002; Sullivan et al., 2003). Here we have demonstrated an important role for T-bet in directing the maturation of the NK and CD1d-restricted Vα14*i* NKT cell compartments as well. In the absence of T-bet, peripheral numbers of NK and Vα14*i* NKT cells are decreased, and the cells that remain appear to be halted at a stage prior to final maturation. While a number of other transcription factors have been demonstrated to regulate NK cell development, little is known about regulatory factors that direct NK and Vα14*i* NKT cell end-stage maturation. We show here that T-bet acts in a cell-intrinsic manner at similar stages in both lineages to complete normal terminal maturation. In its absence, NK and Vα14*i* NKT cell numbers and effector function are diminished. Since NK cells and Vα14*i* NKT cells are derived from different committed precursors and develop in different locations, it appears T-bet acts independently in both cell types after lineage commitment. Given that, compared with mature cells, the expression of T-bet is very low in the progenitor populations of each of these lineages, it is unlikely that it would regulate the formation of these committed precursors.

T-bet$^{-/-}$ NK cells exhibited, on a cell per cell basis, a small but consistent and statistically significant decrease in prototypic target cell killing, consistent with the modest decrease in IFNγ, perforin, and granzyme B, and the known decreased cytolytic ability of immature CD11b^{lo} NK cells compared with fully mature cells (Kim et al., 2002). Similarly, IFN-γ production and NK cytolytic ability to murine cytomegalovirus, a pathogen handled in the mouse primarily by NK cells (Biron et al., 1999), was diminished in T-bet$^{-/-}$ mice. Viral titers in the liver and spleen were unaffected by the absence of T-bet, despite evidence that there are different NK effector mechanisms in operation in these two organs (Tay et al., 1999). This perhaps is not surprising given that the NK cell response against MCMV infection is extremely robust—in vivo depletion of Ly-49 NK cell subsets has minimal or no effect on viral titer in contrast to depletion of the total NK population (Tay et al., 1999). In contrast to NK cells, IFN-γ production by Vα14*i* NKT cells is critically dependent on T-bet, as T-bet$^{-/-}$ cells fail completely to make this cytokine. This failure in IFN-γ production correlates well with the failure of T-bet$^{-/-}$ Vα14*i* NKT cells to reach terminal maturation, the stage at which they downregulate expression of IL-4 and upregulate expression of IFN-γ (Benlagha et al., 2002; Stetson et al., 2003).

Why are there fewer NK and Vα14*i* NKT cells in the periphery in T-bet$^{-/-}$ mice? The explanation likely relates to the maturation defects in both lineages. While the severity of the block in terminal maturation is modest in the NK cell compartment compared with the Vα14*i* NKT cell compartment, the alteration in maturation markers observed indicates that T-bet acts at similar late stages during the developmental maturation of both of these cell lineages. Thus, the absence of T-bet leads to decreased production of Ly-49^{+}CD94^{+}cKit^{lo}α$_{V}$^{lo}CD11-b^{hi}DX5^{hi} mature NK cells, with a significant proportion


A
WT
T-bet-/-
0h
6h
24h
0%
87%
86%
60%
IFN-γ
B
% Specific Lysis
WT
T-bet-/-
Effector:Target Ratio
20 10 5 2.5 1.25 0.625
C
WT
T-bet-/-
40.2%
4%
2.5%
4%
αGalCer Tetramer
IFN-γ
Isotype
D
Serum
Spleen
Liver
IFN-γ (ng/ml of serum)
IFN-γ (ng/g of spleen)
IFN-γ (ng/g of liver)
Days after Infection
E
Day 0
Day 1.5
Day 2
% Specific Lysis
Effector:Target Ratio
F
Log Viral Titer (PFU/g liver)
Log Viral Titer (PFU/g spleen)
Days after Infection

of the peripheral cells present at the penultimate Ly-49$^+$CD94$^+$cKit$^{hi}\alpha_V{}^{hi}$CD11b^{lo}DX5^{lo} developmental stage, as would be observed in the BM of WT mice (Kim et al., 2002). Similarly, NKT cells are blocked at the Vα14*i*$^+$CD-44^{hi}NK1.1$^-$Ly-49$^-$CD122$^-$ stage and do not progress to the fully mature Vα14*i*$^+$CD44^{hi}NK1.1$^+$Ly-49$^+$CD122$^+$ stage. The failure of T-bet$^{-/-}$ Vα14*i* NKT to express significant levels of CD122 and therefore proliferate in response to IL-15 is the simplest explanation for the severe reduction in numbers of this cell type. IL-15 is critically required for peripheral maintenance of NKT cell numbers (Kennedy et al., 2000), and the failure of T-bet$^{-/-}$ Vα14*i* NKT cells to respond to IL-15 (produced normally in the absence of T-bet) would result in impaired expansion and survival in the periphery (Matsuda et al., 2002).

However, impaired IL-15 signaling does not account for the decreased numbers of NK cells, as T-bet$^{-/-}$ NK cells express CD122 and can proliferate in response to IL-15. Immature NK cells at the penultimate stage of development actively proliferate in BM but not in spleen where NK cells remain static (Kim et al., 2002). We have shown an increased basal turnover of NK cells in peripheral lymphoid organs of T-bet$^{-/-}$ mice, consistent with their immature phenotype and reflected by increased expression of the CD69 activation marker. Furthermore, these cells undergo increased spontaneous apoptosis. It is possible that T-bet is required both for maturation and peripheral homeostasis of NK cells so that in its absence cells become spontaneously activated and subsequently die. Alternatively, T-bet could be required for NK cell survival. IL-15 has been shown to be a critical survival factor for NK cells (Minagawa et al., 2002) through its induction of Bcl2, and we have shown that IL-15 induces T-bet in NK cells. However, levels of Bcl2 in T-bet$^{-/-}$ NK cells were normal (unpublished data), and it is currently unclear whether T-bet directly regulates NK cell apoptosis. Another possible explanation for peripheral NK cell deficiency is decreased migration from the BM. Intriguingly, there is a modest increase in CD122$^+$NK1.1$^+$TCRβ^- cells in T-bet$^{-/-}$ BM, and the majority of these cells are CD11b^{lo}. Furthermore, peripheral T-bet$^{-/-}$ NK cells express significantly lower levels of CD43 and integrins shown to be important in cell-cell adhesion and migration (Ostberg et al., 1998; Porter and Hogg, 1998) whose expression correlates with the terminal maturation stage at which NK cells leave the BM and migrate to the periphery (Kim et al., 2002). The decreased expression of integrins and CD43 in the absence of T-bet could therefore result in inefficient NK cell migration to peripheral organs. Further investigation is required to understand mechanisms of NK cell migration, homeostasis, and the function of T-bet in these processes. In any event, it is striking that retroviral overexpression of T-bet resulted in elevated NK cell numbers and restoration of terminal maturation, providing strong evidence that T-bet controls peripheral NK cell homeostasis.

A number of transcription factors that regulate the development of NK and NKT cells, some of them in a stem cell-intrinsic manner like T-bet, have been described (Colucci et al., 2003; Kronenberg and Gapin, 2002). Ikaros acts very early on to regulate mouse lymphopoiesis and homeostasis of the common lymphoid progenitor stem cell, and mice lacking Ikaros have defects in the development of NK cells together with B cells, dendritic cells, and fetal T cells (Boggs et al., 1998; Wang et al., 1996). Deficiency of inhibitors of DNA binding (ID) factor ID2 results in a block in NK cell development due to an inability to form NK precursor cells (Yokota et al., 1999). Members of the ETS family of transcription factors have also been shown to regulate aspects of hematopoiesis in general and NK and NKT cells in particular. PU.1 deficiency results in the generation of reduced numbers of NKP and NK cells (Colucci et al., 2001) while deficiencies in Ets1 and MEF result in severe defects in the development of NK and NKT cells (Barton et al., 1998; Lacorazza et al., 2002; Walunas et al., 2000), but these defects are probably not a result of impaired NK or NKT lineage commitment as with Ikaros and ID2. Further, interferon regulatory factor (IRF)-2-deficient mice demonstrate severe defects in NK maturation and cytotoxicity, but NKT cell development is unaffected (Lohoff et al., 2000). In contrast, T-bet acts in a stem cell-intrinsic manner late in the development of these lineages, consistent with the developmental upregulation of its expression as both cell types mature. We have observed that expression of IRF-2, Ets1, and MEF mRNAs are normal in T-bet$^{-/-}$ NK cells (data not shown), suggesting they act upstream of T-bet. Intriguingly, both the human and mouse T-bet proximal promoters contain two consensus Ets binding sites that are highly conserved in sequence and location (M.J.T., unpublished data). This raises the possibility that ETS family members such as Ets1, MEF, and PU.1 can regu-

Figure 7. NK and NKT Cell Effector Function

(A) WT and T-bet$^{-/-}$ splenic NK cells were stimulated with IL-12 and IL-18. At the times indicated, cells were harvested, fixed, permeabilized, and stained with an antibody against intracellular IFN-γ. Gated NK1.1$^+$TCRβ^- cells are shown. Data are representative of three experiments.

(B) Wild-type and T-bet$^{-/-}$ NK cells were purified by MACS, and equivalent numbers were used as effector cells in standard 4 hr ^{51}Cr YAC1 cell-killing assays. IL-12 and IL-18 were added to all wells. Percentages of specific lysis values for both genotypes were analyzed by Student's t test, and data that were found to be statistically significant (p value of less than 0.05) are marked with an asterisk. Data are representative of three experiments.

(C) Wild-type and T-bet$^{-/-}$ mice were injected intravenously with 2 μg αGalCer. Seventy-five minutes later, liver and splenic (data not shown) Vα14*i* NKT cells were stained with αGalCer tetramer, fixed, permeabilized, and then stained with an antibody against intracellular IFN-γ or an isotype control antibody. Equal numbers of tetramer$^+$ cells are shown in the plots. Data are representative of two experiments.

(D) WT and T-bet$^{-/-}$ mice were infected with MCMV. At the times indicated, levels of IFN-γ were determined in the serum and also in the spleen and liver of the mice by ELISA.

(E) WT and T-bet$^{-/-}$ mice were infected as above, and at the indicated times splenocytes were prepared and used as effector cells in standard ^{51}Cr YAC1 cell-killing assays.

(F) The liver and splenic MCMV titers of the mice infected above were determined by plaque assay at the times indicated. Data are shown as log viral titer plaque forming units (PFU)/gram of liver. All data are representative of two experiments.

late the expression of T-bet during later stages of NK and NKT cell development. GATA-3 has recently been shown to promote NK cell maturation, and GATA-3-deficient NK cells have perturbed cell surface expression of CD11b, CD43, and B220 similar to our observations of T-bet$^{-/-}$ NK cells (Samson et al., 2003). However, GATA-3-deficient NK cells also have abnormal expression of CD94 and certain Ly-49 receptors, suggesting GATA-3 may act earlier than T-bet during maturation. It is intriguing that GATA-3-deficient NK cells express T-bet mRNA at levels 4- to 5-fold lower that WT cells, pointing to a potential requirement for GATA-3 in optimal T-bet induction. The coexpression of T-bet and GATA-3 in NK, Vα14*i* NKT, and $\gamma\delta$ cells clearly contrasts to the mutually exclusive situation seen in T helper cells (Ho and Glimcher, 2002; Yin et al., 2002). Further investigation of GATA-3/T-bet interactions in NK cells is warranted. Collectively, these observations show that, as in T or B cell development, an orderly progression of different transcription factors drives the development of NK and Vα14*i* NKT cell lineages from precursor formation and lineage commitment through acquisition of cell surface receptors and terminal maturation to effector cells.

An observation of interest to us was the difference between the signaling pathways upstream of T-bet in NK as compared to T cells. IFN-γ is directly responsible for the induction of T-bet in lymphoid and myeloid cells (Lighvani et al., 2001) via a STAT1-dependent autoregulatory loop. However, IFN$\gamma^{-/-}$ NK cells had no impairment in T-bet induction by any stimulus tested. Instead, the receptors upstream of T-bet correlated well with the known function of such signaling pathways in NK activation and effector function. Thus, consistent with the multiplicity of signals known to regulate NK cells, multiple signals induced T-bet expression in NK cells and, furthermore, synergized to increase the efficiency of induction. For example, IL-12 in synergy with IL-18, a combination previously described as a potent spur for NK activation, proliferation, and cytokine production (Lauwerys et al., 1999), was the strongest in vitro stimulus placing STAT4 upstream of T-bet in NK cells. Occupancy of IFNγR and TCR results in both T-bet induction and Th1 differentiation. An analogous receptor in NK cells may be the activating Ly-49D receptor that drives cytokine and chemokine production in synergy with IL-12 or IL-18 (Ortaldo et al., 2001; Ortaldo and Young, 2003) since T-bet is synergistically induced by occupancy of Ly-49D together with IL-12 or IFN-α. Of further interest, IL-15 induces T-bet in NK and Vα14*i* NKT cells, thus linking the critical dependence on IL-15 for NK and Vα14*i* NKT development, maturation,and survival (Kennedy et al., 2000; Ranson et al., 2003a) with the induction of T-bet.

We have identified three T-bet target genes in NK cells, perforin, granzyme B, and Runx1, using a combination of chromatin immunoprecipitation and CpG microarray analysis followed by expression studies. The advantage of this system is that it allows identification of direct targets of transcription factors through factor binding to elements in the gene promoter (Weinmann and Farnham, 2002; Weinmann et al., 2002). The identification of perforin and granzyme B as direct T-bet targets is in agreement with two recent reports that T-bet-deficient cytotoxic T cells also express modestly lower levels of granzyme B, and overexpression of T-bet induces perforin expression in developing Th2 cells (Pearce et al., 2003; Sullivan et al., 2003). The latter report also described Eomes, a T-box transcription factor highly expressed in CD8$^+$ T and LAK cells, but not in CD4$^+$ T cells, that also regulates perforin and granzyme B, although direct promoter binding was not shown. The Ets family transcription factor MEF has also been shown to directly control transcription of the perforin gene (Lacorazza et al., 2002). We also confirmed IFN-γ as a T-bet target gene but, interestingly, noted a late but not early deficiency in NK production of IFN-γ in the absence of T-bet. It is possible that Eomes, shown to drive IFN-γ production in CD8$^+$ T cells (Pearce et al., 2003), can compensate for the lack of T-bet at early stages to drive IFN-γ, but that T-bet is required for continued maintenance of the response. Indeed, we have demonstrated that Eomes is expressed at WT levels in T-bet$^{-/-}$ NK cells, an observation that strongly supports this hypothesis. Similar functional redundancy in NK cells has been demonstrated in a study where distinct and redundant kinase signaling pathways downstream of NK cell receptors were found to act synergistically to mediate NK cell effector function (Colucci et al., 2002). The more severe defect in the Vα14*i* NKT than NK compartment might similarly be accounted for by an insignificant role for Eomes in Vα14*i* NKT cells as compared to NK cells. Again, this possibility is strongly supported by our observation of the lack of Eomes expression in the Vα14*i* NKT cell lineage. The lack of Eomes expression in the CD4$^+$ T cell and Vα14*i* NKT cell compartments is suggestive of an important role for Eomes in cytotoxic cells such as CD8$^+$ T cells and NK cells rather than in primarily cytokine-producing cells such as CD4$^+$ T cell and Vα14*i* NKT cells. An intriguing possibility is that the less severe defect in NK cell terminal maturation observed in T-bet$^{-/-}$ mice compared with that seen in Vα14*i* NKT cell development could mean that the expression of Eomes in T-bet$^{-/-}$ NK cells could partially compensate for the absence of T-bet during NK terminal maturation. In the case of Vα14*i* NKT cell development no such compensation would occur as Eomes is not expressed in this compartment, hence the almost complete block in terminal maturation in the absence of T-bet. The differential expression of Eomes in the NK and Vα14*i* NKT cell compartments, together with its possible compensatory role for T-bet, further offers an explanation for the modest defects in cytokine production and cytotoxicity observed in T-bet$^{-/-}$ NK cells compared with the severe defect in IFN-γ production by T-bet$^{-/-}$ Vα14*i* NKT cells. It would be informative to study the development and effector function of NK cells deficient for Eomes and doubly deficient for Eomes and T-bet to further explore a potential role for Eomes in the NK cell compartment. Alternatively, T-bet may partner with different proteins in the two cell types. Overall, it appears that T-bet is one of several parallel acting transcription factors that regulate cytolytic effector molecules in NK cells thereby ensuring rapid, robust, and optimal expression during NK cell effector function.

The Runt family transcription factor Runx1 (AML1, PEBP2αB, or CBFα2) is critically required for hematopoietic stem cell development and T cell differentiation

(Hayashi et al., 2001; Taniuchi et al., 2002). Loss of Runx1 binding sites have been recently associated with susceptibility to autoimmune diseases (Helms et al., 2003; Tokuhiro et al., 2003). The Runx1 gene is regulated in a complex manner, with different splice isoforms being produced from two distinct proximal and distal promoters (Telfer and Rothenberg, 2001). These two promoter elements are utilized in different cell lineages, with the distal promoter active in B and T cells and the proximal promoter active in myeloid and NK lineages. T-bet bound to the proximal promoter, and in T-bet$^{-/-}$ NK cells the expression of Runx1 was decreased. It is currently unclear what significance this has for NK cell development although NKT cell numbers are reduced in Runx1$^{+/-}$ mice (see the Supplemental Data at http://www.immunity.com/cgi/content/full/20/4/477/DC1) and severely diminished in mice that lack Runx1 in the lymphoid system (using Runx1$^{F/F}$/Lck-Cre or Runx1$^{F/F}$/CD4-Cre mice; D. Littman, personal communication). It is intriguing that the proximal isoform of Runx1 has been shown to drive terminal differentiation of a myeloid neutrophil cell line and that enforced expression of Runx1 enhanced the induction of megakaryocytic integrin proteins and cooperated with GATA1 in differentiation of megakaryocytes (Elagib et al., 2003; Telfer and Rothenberg, 2001). Further work is in progress to discern whether Runx1 has a role in NK cell maturation by driving expression of integrins and aiding terminal differentiation.

Taken together, these data demonstrate an important stem cell-intrinsic role for T-bet in NK and Vα14*i* NKT terminal maturation. The fact that expression of transcription factors shown to act earlier during NK cell development (i.e., Ets1, MEF, and IRF-2) is normal in the absence of T-bet suggests strongly that T-bet acts later and possibly downstream of these factors as part of a developmental cascade of transcription factors. This notion is supported by our observation that T-bet expression occurs maximally at late stages of both NK and Vα14*i* NKT development. The expression of Eomes in NK cells but not in Vα14*i* NKT cells points to a cooperative and partially redundant relationship between T-bet and Eomes in NK cell effector function and possibly development. Studies on the interactions between the transcription factors expressed during the development of these lineages should provide further insight into the mechanisms in operation during these processes.

Experimental Procedures

Mice

T-bet$^{-/-}$ mice generated as described (Szabo et al., 2002) were backcrossed eight generations to C57BL/6. WT C57BL/6, Ly5.1 congenic C57BL/6 WT, IFN-$\gamma^{-/-}$, IFN-γR$^{-/-}$, STAT1$^{-/-}$, and STAT4$^{-/-}$ mice were purchased from Jackson Laboratories. Rag2$^{-/-}\gamma_c^{-/-}$ mice were purchased from Taconic Farms. Mice were housed in a specific pathogen-free barrier unit at the Harvard School of Public Health and the University of Colorado Health Sciences Center. Handling of mice and experimental procedures were in accordance with institutional requirements for animal care and use.

Flow Cytometry and Cytokine Assays

Cells were stained with αGalCer/CD1d tetramers as described (Matsuda et al., 2000). mAbs used in this study for flow cytometry include fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-labeled anti-NK1.1 clone PK136, CyChrome-labeled anti-TCRβ clone H57-597, allophycocyanin (APC)-labeled anti-CD44 clone IM7, FITC-labeled anti-CD19 clone MB19-1, PE-labeled anti-CD122 clone 5H4 (eBioscience, San Diego, CA) FITC-labeled anti-Ly-49C/I clone 5E6, FITC-labeled anti-Ly-49G2 clone 4D11, FITC-labeled anti-NKG2A/C/E clone 20d5, PE-labeled anti-CD1d clone 1B1, FITC-labeled anti-CD11b clone M1/70, FITC-labeled anti-CD43 clone S7, PE-labeled anti-CD49b (DX5) clone DX5, FITC-labeled anti-cKit clone 2BE, PE-labeled anti-α_V (CD51) clone H9.2B8, FITC-labeled anti-B220 clone RA3-6B2, FITC-labeled anti-CD69 clone H1.2F3, FITC labeled anti-CD94-NKG2 clone 18d3, and APC-labeled antiCD3ϵ clone 145-2C11 (BD Biosciences, San Diego, CA). Additionally, anti-Ly-49D (clone 4e4) and anti-Ly-49H (clone 3D10) mAbs, kind gifts from Dr. Wayne Yokoyama, were conjugated to Alexa Fluor 488 in our laboratory using standard protocols. For intracellular staining, cells were incubated with blocking 2.4G2 anti-FcγR mAb (eBioscience) and then surface stained. Cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and stained using FITC-labeled anti-IL-4 clone BVD6-24G2 or FITC-labeled anti-IFNγ clone XMG1.2 (eBioscience) according to the manufacturer's protocol. The isotype control used was FITC-labeled mouse IgG1 clone P3 (eBioscience). Cytokines in the serum were evaluated in a sandwich ELISA using rat anti-mouse IL-4 and IFNγ mAbs (BD Bioscience). Apoptosis assays were carried out by costaining with Annexin V and 7-AAD (BD Biosciences) according to the manufacturer's instructions. For in vivo BrdU incorporation assays, mice were intraperitoneally injected with 2 mg of BrdU. After 3 hr, single-cell suspensions were prepared from spleen and bone marrow. Cell were first surface stained with PE-labeled anti-NK1.1 and CyChrome-labeled anti-TCRβ, then fixed, permeabilized, and treated with DNase. Analysis of BrdU incorporation was then performed using the BrdU Flow Kit (BD Biosciences).

Quantitative RT-PCR

Total RNA was extracted from sorted cells with TRIzol solution (Invitrogen, Carlsbad, CA). Reverse transcription was carried out by using the Improm-II reverse transcription system (Promega, Madison, WI) and random primers, or the iScript cDNA synthesis kit (Biorad, Hercules, CA). The amount of amplicon generated during the PCR was monitored by using an Applied Biosystems 7700 (Applied Biosystems, Foster City, CA) apparatus. In some cases, a specific probe labeled with both a reporter and a quencher dye was added into the Taqman PCR mix (Applied Biosystems) at the beginning of the reaction. The sequences of the primers and Taqman probes used in this study have been published (Erlebacher et al., 2002; Gapin et al., 2001; Grogan et al., 2001) or are as follows: mPerforin F, 5′-AAGGTAGCCAATTTTGCAGC-3′; mPerforin R, 5′-GGTTTTTGTACCAGGCGAAA-3′; mGranzymeB F, 5′-TACTGCTGACCTTGTCTCTG-3′; mGranzyme B R, 5′-GATCCTTGATCGAAAGTAAGG-3′; mRunx1 F, 5′-TCAACGACCTCAGGTTTGTCG-3′; mRunx1 R, 5′-GCGGATTTGTAAAGACGGTGA-3′; mRunx1 FAM probe, 5′-CGGAGCGGTAGAGGCAAGAGCTTCA-3′; IL-27 EBI3 F, 5′-ACCCATTGAAGCCACGACTT-3′; IL-27 EBI3 R, 5′-AGTATTGCATCCAGGTGTCAGGT-3′; IL-27 p28 F, 5′-GGCCATGAGGCTGGATCTC-3′; IL-27 p28 R, 5′-AACATTTGAATCCTGCAGCCA-3′.

BM Chimeras and BM Retrovirus Infections

For normal BM transfer experiments, recipient mice were irradiated with 600–800 rads 3 hr before procedure. BM cells were harvested aseptically from the tibia and femurs of donor mice, and 2×10^6 cells were injected intravenously in the recipients. For retroviral infections, BM cells were harvested from donor mice 5 days after they received an intraperitoneal injection of 5 mg 5-fluorouracil (Sigma) in Dulbecco's PBS (Gibco/BRL). The cells were cultured for 4 days at a density of 2×10^6 cells/ml with 20 ng/ml rmIL-3, 50 ng/ml rmIL-6 and 50 ng/ml rmSCF (Peprotech, Rocky Hill, NJ) in DMEM containing 10% FCS. After 48 and 72 hr, the cells were spin infected with control and T-bet-expressing retroviruses generated as described (Szabo et al., 2000). After infections, the supernatant was removed and replaced with fresh media containing cytokines. Recipient mice were irradiated with 600 rads and were then injected with 1×10^6 infected BM cells. In all cases, irradiated mice were maintained on trimethaprim-sulphamethoxazole treated water in sterile cages for 6 weeks before analysis.

In Vitro Culture and Retrovirus Infections

NK cells purified by DX5 MACS or by sorting were cultured in complete RPMI media containing 10% FCS. rmIL-12, rmIL-18, rmIL-15, rmIFN-γ, and rmIFN-α were purchased from R&D Systems and used at the following final doses: IL-12, 10 ng/ml; IL-18, 10 ng/ml; IL-15, 50 ng/ml; IFN-γ, 10 ng/ml; and IFN-α, 20 U/ml. For stimulation of the Ly-49D receptor, 1 μg/10^6 cells of anti-Ly-49D (clone 4E5, a gift from Dr. John Ortaldo) was used at 4°C for 30 min, and the cells were then washed and plated onto a 24-well plate precoated with 10 μg/ml of goat anti-rat IgG (ICN, Aurora, OH) with or without additional cytokines. Cells were cultured for various times, and then utilized for intracellular cytokine staining or harvested for RNA isolation. The effect of IL-15 in vitro was analyzed with CFSE-labeled thymocytes. Thymocytes were plated in 24-well culture plates at a concentration of 2×10^6 cells/well in complete RPMI-1640 medium that contained various doses of recombinant human IL-15 (R&D Systems). Analysis was performed after 3 days in culture. For infection of Vα14*i* NKT cells with retrovirus, freshly isolated thymocytes from T-bet$^{-/-}$ mice were depleted of CD8$^+$ cells and cultured with rmIL-7 (10 ng/ml) for 4 days. The cells were then spin infected with control or T-bet-expressing retroviruses generated as above. After a further 4 days of culture in rmIL-7, the cells were examined using flow cytometry.

NK Cell Killing Assay

NK cell-mediated lysis was measured as release of radioactive isotope from YAC-1 target cells labeled with sodium chromate (^{51}Cr) following incubation with effector leukocytes with or without stimulatory cytokines. Spontaneous release was less than 15% of maximum release. Percent specific lysis was calculated as 100 $\times$ (cpm test sample $-$ cpm spontaneous release)/(cpm total release $-$ cpm spontaneous release).

MCMV Infection

Specific pathogen-free C57BL/6 immunocompetent mice were purchased from Jackson Laboratories (Bar Harbor, ME) or bred from these mice at Brown University. T-bet-deficient mice were shipped from the Harvard School of Public Health and housed at Brown University for 1 week prior to use. All mice were 9 to 10 weeks in age. Mice were infected i.p. with 5×10^4 PFU of Smith strain murine cytomegalovirus and extracted from salivary glands as described (Nguyen et al., 2002). Animals were anesthetized at the indicated times, and blood was collected via the retroorbital route for preparation of serum samples. Mice were sacrificed, and spleens and livers harvested. Splenic leukocytes were obtained using established protocols (Nguyen et al., 2002). Viable cell yields were determined by trypan blue exclusion. Serum IFN-γ levels were determined by standard sandwich ELISA as was previously described (Nguyen et al., 2002). For ELISAs, limits of detection varied between experiments but always were less than 20 pg/ml. Colorimetric changes of enzyme substrates were detected using a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA). To determine MCMV viral titers, serial dilutions of organ homogenates were added to monolayers of NIH 3T3 fibroblast cells (Pien et al., 2000). After 1 week, cells were fixed with 10% buffered formalin. Plaques were visualized with 0.1% crystal violet and quantitated as log PFU per gram tissue. MCMV standards and negative controls were included in each assay.

Chromatin Immunoprecipitation and CpG Island Microarray Analysis

Procedures were carried out essentially as previously described (Weinmann and Farnham, 2002; Weinmann et al., 2002). In brief, chromatin samples were prepared from the human YT NK cell line. The monoclonal mouse anti-T-bet (human and mouse) antibody 3-9D was utilized for these studies (Szabo et al., 2000). Immunoprecipitated chromatin was sheared to an average length of 1–3 kb, and hybridized to a microarray containing 7776 human CpG island clones. Positive clones were confirmed by chromatin immunoprecipitation from the YT cells or from IL-2-expanded, IL-12- and IL-18-activated murine WT and T-bet$^{-/-}$ LAK cells using the 3-9D antibody, followed by PCR against $\sim$300 bp of the proximal promoter for each gene. No signal above background was detected in chromatin from no-antibody control samples or T-bet$^{-/-}$ samples.

Statistics

Data were analyzed using Microsoft Excel software utilizing the two-tailed Student's t test. The null hypothesis was rejected, and differences were assumed to be significant with a p value < 0.05.

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